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Benzodiazepine Derivatives from Marine-Derived *Streptomyces cacaoi* 14CM034

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Abstract: 7-methoxy-8-hydroxy cycloanthranilylproline (**2**), a new natural product with pyrrolobenzodiazepine (PBD) framework, was isolated from marine-derived actinobacterium *Streptomyces cacaoi* 14CM034, together with cycloanthranilylproline (**1**). Structural elucidation of the compounds was based on FTIR, 1D- (^{1}H and ^{13}C NMR), 2D-NMR (COSY, HMBC and NOESY) and HR-MS analyses. Compounds **1** and **2** exhibited notable antimicrobial activity. The presence of PBD derivatives in *S. cacaoi* was first demonstrated with this study.

Keywords: Marine actinobacterium; *Streptomyces cacaoi*; benzodiazepine; antimicrobial activity; antibiotics.

1. Microorganism Source

Actinobacterium 14CM034 was isolated from the sediments that collected from Mersin-Turkey at a depth of 8 m Mediterranean Sea. The strain was identified as *Streptomyces cacaoi* by analysis of the conserved 16S ribosomal DNA region. Primers for amplification of the 16S ribosomal RNA region were 27F and 1492R. The sequence (JX912350.1) obtained was compared pairwise using a BLASTN search and aligned with the sequences of related species retrieved from GenBank, NCBI.

2. Previous Studies

A literature survey ascertained that **1** was a known compound first obtained from a Cruciferous plant *Isatis indigotica* and named as cycloanthranilylproline [1]. This compound was also reported from a mold called *Fuligo candida* in 2004 [2]. Compound **2** was previously synthesized as an intermediate of pyrrolobenzodiazepine-poly(N-methylpyrrole) conjugate synthesis for DNA binding studies [3], benzimidazole linked pyrrolo[2,1-c][1,4]benzodiazepine conjugate preparations for DNA-binding affinity and cytotoxicity studies [4] using chemical methods and as a final product in the development of novel catalyst systems [5, 6].

The previous studies on *S. cacaoi* revealed the presence of polyoxins (nucleoside peptide antibiotics) [7, 8], macrocyclic benzenoid ansamycins, trienomycins [9], polyethers [10] and cyclic peptides [11].

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3. Present Study

A sediment sample, collected from Mersin-Turkey at a depth of 8 m, was used for the isolation of actinobacteria using M6 medium (yeast extract 1g; meat extract 4 g; peptone 4 g; glucose 10 g; NaCl 20 g and agar 20 g in 1.0 L distilled water, pH 7.5) according to the previously described method [12]. Before the molecular characterization, a preliminary fermentation was performed with broth version of the M6 medium at 28 ºC for 14 days at 150 rpm to examine antimicrobial activity. Samples with a volume of 25 mL were taken from the fermentation broth every 4 days from day 2 to 14, were extracted with ethyl acetate (EtOAc) and the activity was monitored by using disc diffusion method on Muller Hinton Agar (MHA) against four different bacteria (*Enteroccocus faecium* DSM 13590: vancomycin-resistant; *Escherichia coli* O157:H7 RSKK 234: streptomycin-, sulfisoxazole-, and tetracycline-resistant; *Staphylococcus aureus* DSM 11729: methicillin-resistant; *Pseudomonas aureginosa* ATCC 27853) and a yeast species (*Candida albicans* DSM 5817). The conserved 16S ribosomal DNA region was used for the identification of the actinobacterial strain showing antimicrobial activity. For this purpose, genomic DNA isolation from cultured strain was performed using the method described earlier [13]. Pairs of universal primers 27F and 1492R were used for PCR amplification. Sequence comparisons were performed using the Basic Local Alignment Search Tool (BLAST) program in the NCBI GenBank database.

An inoculum of culture of *S. cacaoi* 14CM034 was aseptically added to an Erlenmeyer flask (250 mL) containing 50 mL M6 medium, and it was incubated at 28ºC at 150 rpm for 3 days. 5 mL of the culture medium was transferred into 200 mL of M6 liquid medium in 1 L flasks, and further incubation was carried out at 28ºC for 10 days. In total, 50 L of fermentation liquid was obtained after removal of cells by centrifugation. The fermentation broth was extracted with EtOAc three times to yield 4.53 g extract (0.091 g/L yield) after evaporation at 40°C in vacuo. The extract was dissolved in water and partitioned with n-hexane and EtOAc. After evaporation the EtOAc extract (840 mg) was applied to open column chromatography using Polyamide 6 (100 g resin, 100% H_2O to 100% MeOH; 10% decreasing polarity, 1500 mL) to give 292 fractions. Fractions 103-122 were combined to afford compound **2** (13.7 mg). Fractions 53-64 (311 mg) were purified on Sephadex LH-20 (30 g) using MeOH (100%, 300 mL) to afford 81 fractions. Fractions 33-81 were combined to afford compound **1** (12.5 mg).

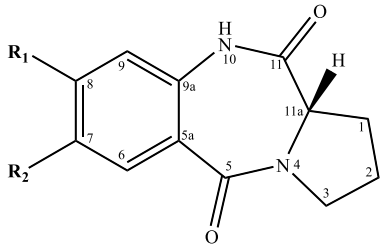
*Compound **1**:* $[\alpha]_D^{23}$ = + 416 (*c* = 1.2, MeOH) [2]; LC-ESI-MS: *m/z* 217.0 $[M+H]^+$ (calcd. for $C_{12}H_{13}N_2O_2$, 217.0977), 238.9 $[M+Na]^+$ (calcd. for $C_{12}H_{12}N_2NaO_2$, 239.0796) and 454.9 $[2M+Na]^+$ (calcd. for $C_{24}H_{24}N_4NaO_4$, 455.1695); ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$): see Table 1.

*Compound **2**:* $[\alpha]_D^{25}$ = + 225 (*c* = 0.04, $CHCl_3$); FT-IR ($CHCl_3$): = 3225, 2923, 1682, 1604, 1437, 1270, 1116, 1020, 785, 731 cm^{-1}; HR-ESI-TOF-MS: *m/z* 263.1037 $[M+H]^+$ (calcd. for $C_{13}H_{15}N_2O_4$, 263.1032); ^{1}H-NMR (400 MHz, DMSO-d_6) and ^{13}C-NMR (100 MHz, DMSO-d_6): see Table 1.

Antimicrobial Activity Assay: Minimum inhibitory concentrations (MIC) of the purified compounds **1** and **2** against *Enteroccocus faecium* DSM 13590, *Escherichia coli* O157:H7 RSKK 234, *Staphylococcus aureus* DSM 11729, *Pseudomonas aureginosa* ATCC 27853 and *Candida albicans* DSM 5817 were performed by the microdilution method described by CLSI [14].

The isolation studies were performed using a Mediterranean sediment sample, which provided a bioactive actinobacterium 14CM034. The isolate exhibited significant antimicrobial activity against methicillin resistant *S. aureus* (18 mm), *E. faecium* (23 mm) and *C. albicans* (14 mm) in the disc diffusion assays. Sequencing using primers 27F and 1492R revealed that the isolate was a member of *Streptomyces g*enus. When the molecular identification evaluated with the phenotypical results, the isolate was identified as *S. cacaoi*, large-scale fermentation of which provided 4.53 g of ethyl acetate

extract. As a continuation of our first study on *S. cacaoi*, which yielded polyether antibiotics [10], further purification studies were performed affording two benzodiazepine derivatives (Figure 1).



1 **2**

$\mathbf{R_1}$ = H $\mathbf{R_1}$ = OH

$\mathbf{R_2}$ = H $\mathbf{R_2}$ = OCH_3

Figure 1. Structures of compounds **1** and **2**

The molecular formula of **1** was determined by LC-ESI-MS (*m/z* 217.0 $[M+H]^+$, 238.9 $[M+Na]^+$ and 454.9 $[2M+Na]^+$), ^1H and ^{13}C NMR data as $C_{12}H_{12}N_2O_2$. The ^1H NMR spectrum of **1** showed the characteristic signals for an ortho-disubstituted benzene ring (δ 7.04, 1H, dd, J=1.2 and 8.0 Hz; δ 8.01, 1H, dd, J=1.6 and 8.0 Hz; δ 7.27, 1H, dt, J=1.2 and 8.0 Hz; δ 7.47, 1H, dd, J=1.6 and 8.0 Hz). The resonance of one of the aromatic dd signals at 8.01 ppm indicated the presence of an electron withdrawing group in the ortho position, while other dd signal at 7.04 ppm indicated a substitution of an electron donating group in ortho position. In addition, a broad singlet of proton at 8.68 ppm was attributed to an N(H) proton. The ^{13}C NMR spectrum of **1** showed signals for two carbonyl carbons (δ 165.6 and 171.5), along with six resonances (four methine and two quaternary carbons) of the o-disubstituted benzene ring between 120 and 140 ppm in the low field. The chemical shift of these carbons indicated that they were part of an ester or amide functional group. The presence of two oxygen atoms in the molecular formula of **1** as carbonyl (C=O) readily implied the presence of two amide groups. Moreover, 3 methylene carbons (δ 23.7, 26.5 and 47.5) and one methine carbon (δ 56.9) were noted in the high field region of ^{13}C NMR spectrum. When the spin systems of **1** were deduced from the COSY spectrum, a $-CH_2-CH_2-CH_2-CH-$ unit was determined in addition to the aromatic system. The interfragment relationship deduced from the HMBC spectrum verified that substitutions on the aromatic ring were through the carbonyl carbon [-C(=O)-N=] ($^3J_{C\text{-}H}$ from C-5 to H-6 correlation) and the nitrogen atom (O=C-NH-) ($^3J_{C\text{-}H}$ from C-5a to NH). The HMBC spectrum also provided key correlations between the second spin system and the amide functionalities including C-5 (δ 165.6) to H-11a (δ 4.09) and C-11 (δ 171.5) to H-1a (δ 1.99 – 2.09 m) revealing a pyrrolobenzodiazepine framework (Figure 2). In regard to stereochemistry of C-11a, the biosynthetic origin of the pyrrole ring (L-proline) and all the pyrrolobenzodiazepine derivatives isolated from nature were taken into consideration suggesting 11a(S) configuration [15, 16].

The HR-ESI-MS analysis of **2** (*m/z* 263.1037 $[M+H]^+$) suggested a 46 amu increase compared to compound **1**. ^1H and ^{13}C NMR spectra indicated that compound **2** was also a pyrrolobenzodiazepine. It was readily deduced that the high field carbon and proton signals of the pyrrole unit were similar in two compounds, whereas the difference was mainly due to the aromatic resonances including an aromatic methoxy group. The ^1H NMR spectrum of **2** revealed a 1,2,4,5-tetrasubstituted benzene system based on two singlet resonances observed at δ 6.54 and 7.20. The presence of a methoxy group was evident from a proton signal at δ 3.76 in addition to a carbon resonance at δ 57.0 in the ^{13}C NMR spectrum, which implied that an extra aromatic –OH group was required to explain the mass difference (46 amu) between compounds **1** and **2**. The position of the methoxy and hydroxy groups together with the amide substitutions were established by long distance correlations in the HMBC spectrum and 2D-NOESY spectra. The cross HMBC peak from C-5 (δ 165.2) to H-6 (δ 7.20), and the NOESY correlation between N(H) (δ 10.15) and H-9 (δ 6.54) were evident for locating of the amide functionalities. Additionally, the strong $^3J_{C\text{-}H}$ correlations from C-7 to the O-methyl resonance (δ 3.76) and H-9 (δ 6.54), C-8/C-9a (δ 151.0 and 131.7, respectively) to H-6 (δ 7.20) and the NOESY correlation between H-6 (δ 7.20) and *O*-methyl resonance (δ 3.76) confirmed the positions of oxygen substitutions (Figure 2). On the basis of

biogenetic perspective, the absolute configuration of C-11a was also proposed as S as in **1**. Thus, the chemical structure of **2** was elucidated as 11a(S),7-methoxy-8-hydroxy-1,2,3,11a-tetrahydro-5H-benzo[e]pyrolo[1,2-a][1,4]diazepine-5,11(10H)-dione.

Table 1. ^{13}C and ^{1}H NMR data of **1** (in $CDCl_3$) and **2** (in DMSO-d_6) (100 and 400 MHz, respectively).

C\H	**1**		**2**	
	δ_C (ppm)	δ_H (ppm), *J* (Hz)	δ_C (ppm)	δ_H (ppm), *J* (Hz)
1	26.5	2.78 m, 1.99 – 2.09 m*	26.4	2.45 m, 1.91 m
2	23.7	1.99 – 2.09 m *(2H)	23.8	1.76 m, 1.85 m
3	47.5	3.81 m, 3.62 m	47.4	3.51 m, 3.41 m
5	165.6		165.2	
5a	127.4		118.1	
6	131.4	8.01 d (1.6, 8.0)	108.5	7.20 s
7	125.3	7.27 dt (1.2, 8.0)	145.2	
8	132.6	7.47 dt (1.6, 8.0)	151.0	
9	121.3	7.04 d (1.2, 8.0)	113.1	6.54 s
9a	135.5		131.7	
10		8.68 br s		10.15 s
11	171.5		171.0	
11a	56.9	4.09 d (4.0)	56.4	4.01 d (4.0)
-OCH$_3$			57.0	3.76 s

*overlapped resonances

Compounds **1** and **2** are PBD derivatives formed by conjugation of anthranilic acid and L-proline [2]. PBDs are either naturally produced by actinomycetes or synthetically produced [17]. The first one was anthramycin, discovered in 1963. To date, 16 of the PBDs isolated from *Streptomyces* and *Micrococcus* were found to have moderate antimicrobial activity [18]. The main reason for this group of compounds gaining importance is the specific binding to the guanine units in the DNA minor groove and being highly effective DNA alkylating agents [19,20]. Thus, some PBD derivatives were shown to be strongly cytotoxic against some cancer cell lines at pM and nM levels (eg. cyberomycin, tomaymycin, anthramycin) [21]. Many synthetic and semi-synthetic derivatives of this group of compounds have been prepared and studied thoroughly, and some members went under clinical trials (eg. SJG-136, pyrrolobenzodiazepine dimer) [22].

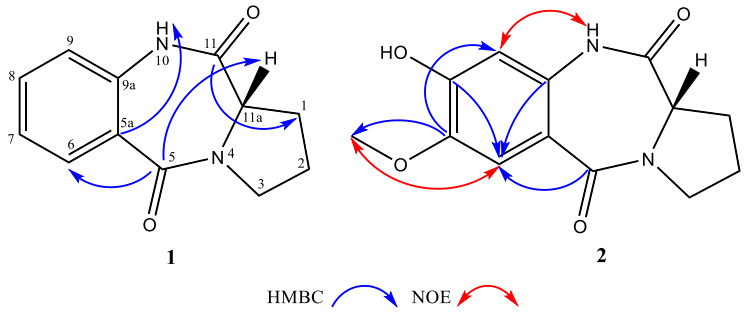


Figure 2. Key HMBC and NOE correlations of compounds **1** and **2**

The functional group at C-11 was shown to be important in both antimicrobial and cytotoxic activities. The presence of an oxo group in the C-11 position greatly reduces efficiency [18]. In parallel to those studies, compounds **1** and **2** with 11-oxo functionalities showed no cytotoxicity in our tests versus cancer cell lines (>50 µM; Caco-2, MCF-7, U87MG, HeLa, PC3, Vero). However, MIC values were found to be ranging from 8.75 to 32 µg/mL (Table 2).

Table 2. MIC values of compounds **1**, **2** and positive controls against the test organisms

Compounds	MIC (µg/mL)				
	E. coli **O157:H7 RSKK 234**	***MRSA*** **DSM 11729**	***E. faecium*** **DSM 13590**	***P. aeruginosa*** **ATCC 27853**	***C. albicans*** **DSM 5817**
1	16	32	16	16	16
2	8.75	17.5	8.75	8.75	8.75
Lincomycin	64	-	-	-	-
Nalidixicasid	4	32	-	-	-
Azithromycin	16	-	-	-	-
Cefaclor	8	32	-	-	-
Ceftriaxone	-	-	-	-	-
Clindamycin	16	4	-	-	-
Tetracycline	8	4	-	-	-
Ampicillin sulbactam	32	-			
Amphotericin B					0.5
Fluconazole	-	-	-	-	8

-:Not tested

In conclusion, *Streptomyces* genus is one of the richest sources of bioactive compounds with wide chemical diversity. Herein, we report pyrrolobenzodiazepine derivatives with antimicrobial activity for the first time from *S. cacaoi*. Further studies are in progress to obtain new molecules from *S. cacaoi* via medium optimization.

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Supporting Information

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